HIGH SENSITIVITY DETECTION OF BACTERIAL ENDOSPORES VIA TB PHOTOLUMINESCENCE ENHANCEMENT

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ABSTRACT

Detecting bacterial endospores is a critical challenge to bioanalytical chemistry, since a number of serious diseases and health problems are caused by members of the spore-forming genera *Bacillus* and *Clostridium*. We have developed a highly sensitive method for their detection and have demonstrated detection limits of less than 5000 CFU/ml. Our method is based on the presence of a marker compound in bacterial endospores, dipicolinic acid (dpa). When complexed with Tb and excited in the UV, the dpa enhances the photoluminescence emission of Tb by several orders of magnitude.

We have investigated the potential for interference from other biological materials and chemicals and found that nothing other than bacterial endospores will give us a positive response to this test. Our investigation also showed that the presence of phosphate or organophosphate ions will reduce the observed signals. We have been able to overcome this problem through the addition of $AlCl_3$. The results of our interference studies and phosphate studies will be presented.

Since only 10% or less of the dpa is released when the endospores are suspended in aqueous buffer, we have also examined methods for enhancing the release of dpa. Our results from both mechanical and chemical methods to enhance dpa release will be presented. The best we have achieved is a 20-fold increase in dpa release from *B. globigii* endospores in 2 minutes through the addition of dodecylamine and heating to 80°C.

INTRODUCTION

The detection of bacterial endospores is a significant challenge in bioanalytical chemistry. The endospore is a dormant stage in the life cycle of some members of the genera *Bacillus* and *Clostridium*. A number of these species can cause disease, food poisoning, or food spoilage, so their detection is important in both the civilian and military sectors. The method we describe in this paper is intended for first-alert use. In this intended deployment the method is

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used to monitor the background and when the signal level increases significantly, an alert is issued to trigger other more specific and time-consuming techniques.

Our method is based on the presence of calcium dipicolinate (Ca(dpa)) in the endospore casing and the release of some of this dpa into solution. Endospores contain 2 to 15% by weight[1] of dpa,[1] depending on species and other factors. Addition of Tb^{3+} to the aqueous solution will result in complexation with the dpa^{2-} released from the spore. When irradiated with UV light at the dpa absorption maximum, $[Tb(dpa)_n]^{3-2n}$ exhibits a greatly enhanced luminescence compared to Tb^{3+} alone. The observed luminescence is shifted far from the excitation wavelength and is narrower than normal molecule luminescence signatures. Detection of the Tb^{3+} luminescence signature is therefore a unique marker for the presence of dpa and thus bacterial endospores. In our experiments we use an excess of Tb^{3+}, so the form of the complex is $[Tb(dpa)]^{+}$. Previous research has determined that energy transfer from the ligand (dpa^{2-}) to the terbium excited states leads to this enhancement.[2,3] In this method, a small amount of an aqueous suspension of the analyte is added to a buffered $TbCl_3$ solution. Any samples containing particulates are filtered to isolate the water-soluble compounds. The sample is then irradiated with a wavelength corresponding to the dpa absorption maximum and the luminescence emission spectrum is collected. Any sample that exhibits stronger emission intensity than Tb^{3+} alone contains bacterial endospores.

Our research has been divided into three major studies. First was the examination of various chemical and biological materials for interference with our detection method.[4] This involved investigating both false positive and negative results. False positive testing checked for responses for samples that do not contain bacterial endospores. False negative testing investigated cases where the reduction or elimination of signal from samples containing bacterial endospores occurred. The second study concerned finding an additive to reduce the deleterious effect of phosphate, the primary interferent with our technique.[6] Finally, we have investigated methods to release more dpa from the bacterial endospores in order to improve the limit of detection. In this phase of our work we examined several mechanical and chemical methods to extract more dpa.

EXPERIMENTAL

The experimental procedures and optical arrangement are described in detail in our previous publications.[4-6] The samples in each group of experiments are brought to a constant volume with buffer and the level of Tb and *B. globigii* are held constant within each set to permit proper comparisons. All of the samples were made in aqueous Trizma buffer at pH 7.6. Three replicate measurements are made on each sample and the average value for the three replicates is used for succeeding calculations and in all plots. The signal from each set of samples is normalized to a standard with the same bacterial endospore concentration and total volume.

RESULTS AND DISCUSSION

INTERFERENCE STUDY. We have examined materials in three classes to determine their effect on our bacterial endospore detection technique.[4, 5] We checked nine organic chemicals, seven inorganic salts, and fourteen biological materials for both false positive and false negative responses. A false positive occurs when a material other than a bacterial endospore give a signal above the Tb background. None of the materials we examined gave a

false positive signal. In fact, the only materials that yielded a positive response were bacterial endospores, as seen in Figure 1. Figure 1 includes the responses for two samples of *B. globigii*

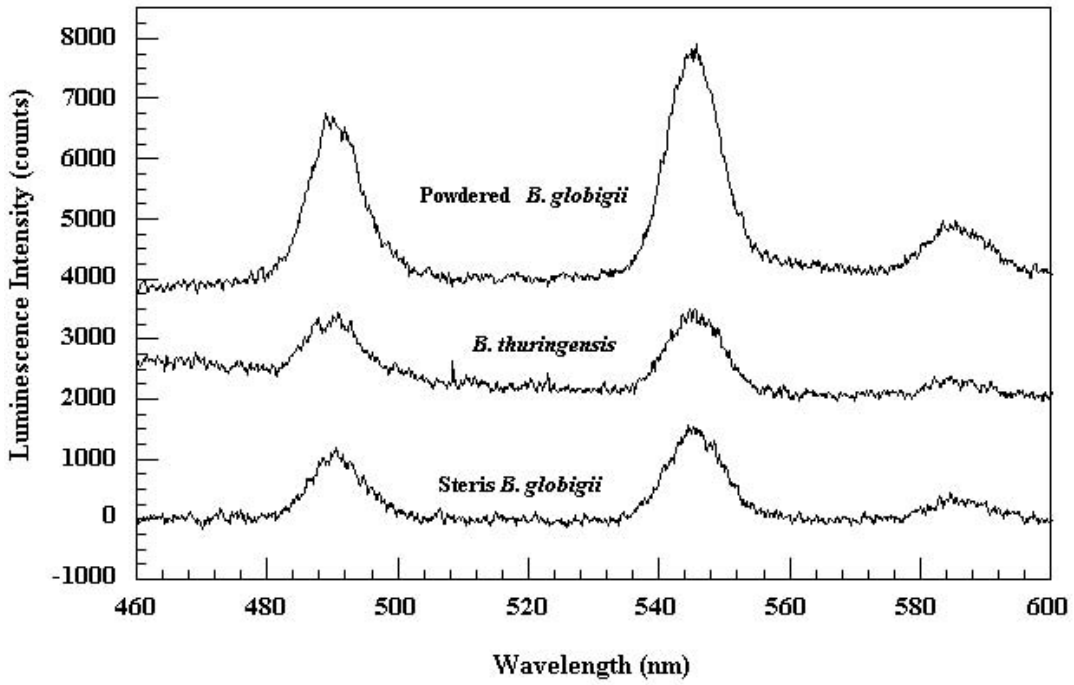

Figure 1. Positive responses from bacterial endospores. Top-powdered *B. globigii* 6.165×10^8 CFU/mL, intensity offset by + 4000; Middle-Thuricide®, containing 0.8 % *B. thuringensis*, diluted by a factor of 40, intensity offset by + 2000; Bottom-Steris *B. globigii* 1.3×10^9 CFU/mL.

and a commercial insecticide containing 0.8% *B. thuringensis*. The Steris *B. globigii* was washed by centrifugation and we were still able to detect the signal from the dpa remaining in the spores.

Several of the materials in the test set did result in reductions in the signals we observed from a fixed amount of *B. globigii*. Some difficulties were also observed with a number of the pollens and molds, but these materials can be removed through size-selective sampling since they are much larger than bacterial endospores. The worst case occurred with addition of phosphate-containing chemicals. K_2HPO_4 had the most deleterious effect on our signals. Barela and Sherry have reported that phosphate buffer has a similar inhibiting effect on the photoluminescent emission from terbium dipicolinate.[7] Two explanations are possible for this observation. The first is that the phosphate anion has a high affinity for Tb^{+3} and may displace dpa from it. Secondly, the phosphate anion may quench the excited dpa molecules before they transfer their energy to the Tb^{+3}. These results demonstrated the need for a method to deal with phosphate interference.

PHOSPHATE INTERFERENCE. Our next set of experiments[6] focused on finding a method to reduce or eliminate the deleterious effect of phosphates described in the previous section. We found that *B. globigii* samples from Dugway Proving Ground were less susceptible to phosphates than dpa alone. It is possible that this resistance is due to the additional

components[8] in those samples as a result of their growth and processing. Despite the additional "protective" components, samples of Dugway *B. globigii* still experienced a decrease in signal of up to 15% when exposed to 85 μM K_2HPO_4. At the same concentration of K_2HPO_4, dpa alone suffered up to a 65% decrease in signal strength.

The next step in our investigation was to examine the use of various inorganic salts to reduce the interference of phosphates. Figure 2 shows the results of this examination. We

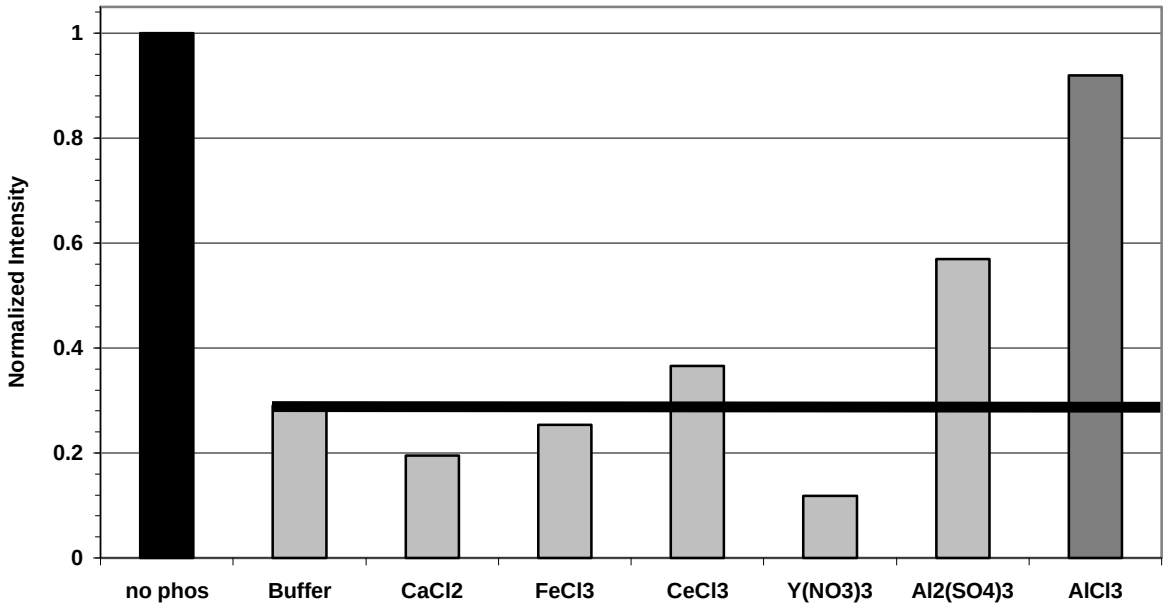

Figure 2. Signal recovery on addition of salt solutions. The signal is normalized to the intensity (no phosphate) of the Tb/dpa stock with buffer added to compensate for the volume of salt solution added. Buffer represents the normalized signal level of the Tb/dpa/phosphate stock with buffer added to compensate for the volume of salt solution added. The beneficial effects of $Al_2(SO_4)_3$ and $AlCl_3$ are clear.

concentrated on salts with cations with a +3 charge. It has been reported that Al^{+3} is used in water processing facilities to remove phosphate ions.[9] The utility of this approach is clearly evident from these results. The salts with anions containing a negatively charged O were not effective and served to reduce the signal or hinder the beneficial effects of the cation. This is a direct result of the high affinity of Tb^{+3} for negatively charged oxygens.[2, 3] When $AlCl_3$ was added to samples containing dpa or Dugway *B. globigii*, the signal level was reduced by up to 15% for dpa and maintained for the Dugway *B. globigii* on exposure to 85 μM K_2HPO_4, provided that the dpa was added before or at the same time as the phosphate. This graphically shows the beneficial effect of addition of $AlCl_3$.

DPA RELEASE ENHANCEMENT. We have focused our most recent efforts on determining the best method of enhancing the release of dpa from the bacterial endospores, since less than 10% of the available dpa is released by suspension in aqueous buffers. In this study we used a washed commercial suspension of *B. globigii* that had a significantly lower level of readily available dpa upon suspension in aqueous buffer than the Dugway *B. globigii* did. We have examined both mechanical and chemical means of extracting more dpa from the endospores. Table 1 summarizes the results of these experiments.

TABLE 1. Summary of DPA release methods. The maximum enhancement is the ratio of the maximum signal achieved and the room temperature standard with the same concentration of bacterial endospores.

Method	Maximum Enhancement	Time to Max. Signal
Glass Beads	6.3	2 min.
Diatomaceous Earth	4.8	12 min.
Cetyltrimethylammonium bromide	7.4	120 min.at 50°C
Amino Acid/Sugar	5.8	180 min. at R.T.
Dodecylamine	20.5	2 min. at 80°C
Boiling	18.3	15 min. at 100°C

Among the mechanical means we have attempted are sonication, sonication with glass beads (40 nm and 200 nm), shaking with glass bead and diatomaceous earth, and heating. Sonication and shaking with glass beads was intended to crush the endospores between the glass beads. Shaking with diatomaceous earth abraded the endospores on the sharp facets of the diatomaceous earth. It has been reported in the literature[1, 10] that boiling endospores for 15 minutes will extract all of the dpa from them. We have found that sonication with or without glass beads was ineffective on our samples. Agitation in a Wig-L-Bug with glass beads or diatomaceous earth increased the extracted dpa by a factor of 6.3 and 4.8, respectively. One of the difficulties with the diatomaceous earth required 10-15 minutes of settling time after being agitated in order to extract a sample of the suspension.

We also examined three chemical means of extracting more dpa. The first was to mix a solution of cetyltrimethylammonium bromide (CTAB) with a sample of the bacterial endospore suspension and heat the mixture to 50°C. Samples were removed at set time intervals, mixed with Tb solution, and the photoluminescence emission collected. This method took two hours to reach its maximum release and increased the dpa by a factor of 7.4. We also attempted to coax the endospores into germinating, since the first step in that process is the expulsion of all of the dpa from the endospore. This was accomplished by adding a mixture of alanine, asparagine, and glucose to a sample of the endospore suspension, sampling at intervals, and collecting the photoluminescence emission. This method released 5.8 times the initial dpa concentration and took three hours. Later experiments, in which the endospores are pre-heated, have achieved almost complete release of dpa from the spores with either alanine alone or the alanine-asparagine-glucose cocktail, but it still required 2 hours to 3 hours to reach the maximum level of dpa release. Neither of these methods resulted in acceptable release rates. The last chemical method we have examined is addition of dodecylamine (DDA) to endospores suspensions. This was done in a similar manner to the CTAB addition. The DDA was able to release all of the dpa (as determined by boiling) from the bacterial endospores in 2 minutes at 80°C. The dpa release with DDA is a strong function of relative endospore-to-DDA concentration and temperature.[11] Once a threshold level of DDA is reached, the rate of dpa release is independent of endospore-to-DDA ratio. The rate also increases as a function of temperature, as one would expect. Clearly, DDA addition is the best method for releasing more dpa from bacterial endospores.

DISCUSSION. The release of all of the dpa from the endospores permits the most sensitive possible detection of bacterial endospores with this technique. Based on our un-

enhanced limit of detection of 83,000 CFU/mL of suspension for *B. globigii*,[11] we can detect less than 5000 CFU/mL with DDA release. We have experimentally detected less than 10,000 CFU/mL of BG using the DDA treatment with a signal-to-noise ratio of greater than 6, indicating that our LOD will be less than 5000 CFU/mL. This level of detection permits detection at the 10 agent-containing particle per liter of air level in less than 10 minutes of sampling at the 500 L/minute rate and concentration to 1 mL. Commercial samplers with this rate of collection are available permitting extremely sensitive detection of bacterial endospores from the atmosphere. With a commercial fluorescence system and no processing, Dugway *B. globigii* has been detected at a level of 1900 CFU/mL.[12] Improvements in our system may permit us to reach this level of detection and with the DDA treatment reach detection limits of less than 200 CFU/mL. One area of concern to quantitative use of this technique is the reproducibility of sampling from a suspension. This results in a 15% relative standard deviation for replicate samples[6] and limits quantitative use of the method. Despite this limitation, our technique is well-suited to use as a first alert warning system.

CONCLUSIONS

The results we have reported clearly indicate the high sensitivity of our method for bacterial endospore detection. We are able to detect less than 5000 CFU/mL of endospores in less than 5 minutes with DDA for enhanced release of dpa from the endospores. Our method is immune from false positive responses. Through the use of $AlCl_3$ to complex any phosphate present in a sample, we have eliminated the only significant interference with this technique. While quantitative use of the method is limited, it is more than sufficient for first-alert use.

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